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(54) Title: MULTI-LABEL TIME-RESOLVED FLUORESCENCE ANALYSIS OF NUCLEIC ACID SEQUENCES USING LANTHANIDE CHELATES

(57) Abstract

The invention pertains to a method for simultaneous real time fluorometric sequence analysis of nucleic acid fragments of different lengths and four different terminals, the fragments being labelled with different fluorescent labels for the respective terminating bases. The fragments are labelled with four different labels comprising the same two lanthanides and the same two ligands as follows: label 1 = ligand 1 + lanthanide 1, label 2 = ligand 2 + lanthanide 1, label 3 = ligand 1 + lanthanide 2, label 4 = ligand 2 + lanthanide 2, where ligands 1 and 2 have different excitation wavelengths and lanthanides 1 and 2 have different emission wavelengths, excitation light is generated in the form of light pulses alternating between the different excitation wavelengths of the ligands, and the photons emitted by the labels are detected periodically for the different emission wavelengths of the lanthanides.

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Multi-label time-resolved fluorescence analysis of nucleic acid sequences using lanthanide chelates

The structural analysis of nucleic acid has a central role in molecular biology. The presently available techniques for nucleic acid sequence analysis are laborious and expensive and often involve the use of radioisotopes. For this reason, and for sequencing an endless number of genes including the human genome with 3×10^9 bases, the development of an automated non-isotopic method for nucleic acid sequence analysis is extremely important.

The commonly used sequencing methods are based on the work by Sanger et al. (Sanger, F., Nicklen, S. & Coulson, A.R., 1977, Proc. Natl. Acad. Sci. USA, 74: 5463-5467). Using an enzymatic method DNA fragments terminating in either adenosine (A), cytidine (C), guanosine (G) or thymidine (T) can be produced. Another method of importance was developed by Maxam et al. (Maxam, A.M. and Gilbert, W., 1980, Meth. Enzym. 65: 499-559). In this chemical method fragments terminating in G, G + A, C + T and C are produced. In both cases these four sets of DNA fragments with different lengths are then separated by electrophoresis in adjacent lanes in a high resolution polyacrylamide gel. The image of the gel can then be examined by a traditional autoradiographic technique.

Obviously a more cost effective method for automatic real time reading of electrophoresis scans is fluorescence labeling and the use of an appropriate fluorometric system for each electrophoresis lane. Conventional fluorescent probes including fluorescein or tetramethylrhodamine are applicable. This method, however, requires four independent excitation and detection systems and is clumsy and expensive. The use of Argon ion laser with beam splitters for the excitation is advantageous and improves the sensitivity. Recently Smith et al. (Smith L.M., Sanders, J.Z., Kaiser, R.J., Hughes, P., Dodd, C., Connell, C.R., Heiner, C., Kent, S.B.H. & Hood, L.E. 1986, Nature, 321: 674-679) presented an automatic sequence reader device. This system was based on the use of four organic fluo-

rescent probes (fluorescein, NBD, tetramethylrhodamine and Texas red) one for each terminal (A, C, G and T) and the labeled DNA fragments were electrophoresed in the same column. An Argon ion laser fluorescence detector was used for real time monitoring of the DNA bands during the electrophoresis. Fluorescence signals of each color indicated the DNA bands and the information was directly stored in the computer.

In the system described by Smith et al. (1986) the advantage of the method resided in the possibility to use a single column electrophoresis system, but the use of four traditional fluorescent probes with strongly overlapping excitation and emission spectra required Argon ion laser excitation and advanced computer data reduction in order to eliminate signals caused by the interference of other fluorescent probes. The laser excitation was useful also for reducing the background scatter originating from the gel and from the associated circular glass tubing. The employment of Argon ion laser increases the system cost considerably.

The strategy of the present invention is to provide an automatic real time nucleic acid sequence analyser for a single column electrophoresis system using four fluorescent probes for a simultaneous read-out of each of the four base specific reactions. The nucleic acid fragments generated in each reaction are identified on the basis of the specific excitation and emission wavelengths of the fluorescent probes. The conventional fluorescent probes, however, have strongly overlapping excitation and emission spectra as shown in the work of Smith et al. In order to overcome this problem it was decided to investigate the possibility of using fluorescent lanthanide chelates in automatic real time nucleic acid sequencing.

Lanthanide chelates have become a very promising alternative as labeling or dye material for biospecific assays and cell analyses (Hemmilä, I., 1985, Anal. Chem. 57: 1676-1681; Hemmilä, I., Dakubu, S., Mukkala, V-M., Siitari, H. and Lövgren, T. 1984, Anal. Biochem. 137: 335-343). A general problem in multi-parameter analysis, however, is the availability of a sufficient number of different lanthanides with different emission wavelength bands. Europium and terbium have emission bands at 614 nm and 545 nm, respectively, and they also provide relatively strong fluorescence when they form appropriate chelates. Samarinium has its specific emission

band at 640 nm, which can be efficiently separated from the emission of Eu and Tb, and consequently the use of Eu, Tb and Sm allows a three parameter analysis in the same sample. The fluorescence emission of the other lanthanides, however, is too weak or their chelates currently known are not stable in water solutions. Therefore, it is difficult to find for example four lanthanides with different emission wavelengths for multi-parameter analysis using four labels in the same sample.

This invention is related to the discovery of new chelate structures which include an active functional group for covalent binding to modified nucleic acid fragments and which almost equally bind europium and terbium but have different excitation wavelengths. As an example, ligand A has the excitation maximum at 274 nm and ligand B at 307 nm. The following combinations of four different lanthanide chelates are then useful for analysing the sample with four parameters.

Chelate	Excitation wavelength	Emission wavelength	
Ligand A - Eu	274 nm	614 nm	Compound 12
Ligand B - Eu	307 nm	614 nm	Compound 24
Ligand A - Tb	274 nm	545 nm	Compound 13
Ligand B - Tb	307 nm	545 nm	Compound 25

The band widths of the excitation and emission spectra of these chelates - as shown in Figures 1-4 - are narrow enough for the detection of each of these four chelates in the same sample using a filter fluorometer. In actual practice, this is achieved by changing the excitation and emission wavelengths according to the above table, and recording four signals for each measurement. In real time DNA sequencing the recording of each of the four DNA terminals (A, C, G and T) directly from the electrophoresis in the same column can be carried out using an appropriate double excitation/double emission wavelength fluorometer where recording of each wavelength combination is fast enough when compared with the movement of the DNA bands in the electrophoresis gel.

As demonstrated in Figure 1, the emission peaks of Tb and Eu at 544 nm and 614 nm, respectively, are sharp enough without any significant overlapping and interference for almost complete separation of the signals. In Figures 2 and 3 it can be seen that the excitation efficiencies of chelates with ligand A are very low at 307 nm. In Figures 4 and 5 it can be seen that the excitation efficiencies of chelates with ligand B at 274 nm are about 25 % of the maximum at 307 nm. This gives an interference which, however, is low enough for reliable data reduction.

Naturally, it is possible that some other ligands may provide even lower excitation efficiency at 274 nm or any other suitable wavelength, and consequently the interference between the signals is lower and no computer capacity is needed for interference reduction.

Besides the selectivity, the sensitivity is of importance in the detection of nucleic acid bands from electrophoresis. The most important sources of the background of biochemical and biological samples are the "autofluorescence" and the scattering. The light emitted from these sources appears simultaneously with or promptly after the excitation as the fluorescence decay time of bio-organic substances is very short. The use of lanthanide chelates as fluorescent probes offers a possibility for efficient reduction of the background and scattering because the fluorescence decay time of lanthanides is long. Time-resolved fluorometry efficiently discriminates the prompt background from the long decay fluorescence of the probe because measurement does not occur until a certain time has elapsed from the moment of excitation. Thus it offers an almost ideal way of measuring probes with long fluorescence decay and considerably improves the sensitivity compared with the conventional measurement methods with short decay time organic fluorescent probes.

Lanthanides and their chelates are a special group of compounds which display ion fluorescence with decay times in the order of 50 to 1000 µsec. and these compounds are very useful for time-resolved microsecond fluorometry. In comparison to the best organic fluorescent compounds the lanthanide chelates can provide comparable, or better overall sensitivity when used in time-resolved fluorescence detection (Soini & Kojola, Clin. Chem. 29, 65, 1983). This is the case despite the lower quantum yield which is around one tenth or less that of the best organic fluorescent compounds. The low quantum yield stems largely from the wide Stokes shift and the long decay time of the fluorescent signal.

High detection sensitivity can be achieved with a simple fluorometric system based on the use of a Xenon flash lamp as an excitation source and interference filters for selecting the proper excitation and emission bands. Small Xenon flash lamps with a pulse duration of 1 µs are commercially available and well suited for this purpose.

The operation of the time-resolved fluorometric system is shown schematically in Fig. 6. The light source is pulsed having a width of t_p. The signal has a maximum instantaneous intensity I_p at the end of the excitation pulse. The excited states of the fluorescent label relax with a decay time τ and after a suitable delay time t_d the signal from the detector is sampled for a certain period of gate time, t_g. The excitation is repeated at repetition frequency f and the time interval between the pulses $t_r = 1/f$. The fluorescence signal will be integrated during the gate time t_g over a definite number of measurement cycles (n).

Instrumentation which is available and applicable to time-resolved fluorometric studies with lanthanide chelates should be equipped with a pulsed light source producing very short light pulses in comparison with the actual decay times. The interferences between different fluorescent complexes can be reduced by optimizing the delay time t_d and gate time t_g, because the complexes have different decay times τ. Therefore, in addition to optimizing the excitation and emission bands in multiparameter analysis, it is important to control the t_d and t_g. The control should be made automatic and synchronous with the wavelength control.

Figure 7 illustrates the principle of the nucleic acid sequencing device according to the invention. The electrophoresis column (3) communicates with an upper buffer reservoir (1) and a lower buffer reservoir (2). The excitation light sources producing light pulses of wavelength 274 nm (5) and wavelength 307 nm (6) incorporate an appropriate interference filter and lens system. The light pulses are focused in point (4) in the column (3). Fluorescence emissions from the column are collected to the detectors (7) and (8). The detectors include an appropriate lens system and interference filters for wavelengths 544 nm and 614 nm.

The excitation light sources and detectors are controlled by the electronics unit, and activated periodically for example as follows

1st cycle	light source 5	detector 7
2nd cycle	light source 5	detector 8
3rd cycle	light source 6	detector 7
4th cycle	light source 6	detector 8

in such a way that emissions at both wavelengths are recorded for both excitation wavelengths and these 4 cycles are repeated until a sufficient

number of photon counts has been recorded for acceptable statistical precision. The electronic control system is also adjusted to record the fluorescence emission in a time-resolved mode.

Because the constructions of the flash light sources and detectors are relatively simple, the employment of two separate units for both wavelengths does not increase the cost of the system significantly. Naturally, single units with appropriate chopper systems for the interference filters can also be used.

The wavelengths given above for ligands A and B and for terbium and europium are presented as examples. The invention is not limited to the use of terbium and europium as a pair, or ligand A (274 nm max excitation) and ligand B (307 nm max excitation) as a pair, but any other pairs with appropriate wavelengths are within the scope of this invention.

The invention is defined in the claims that form a part of the descriptive part of this specification. The invention will now be illustrated by means of examples. The formulae and synthetic routes for the chelates employed are shown on scheme 1-3.

Example 1 (Scheme 1)
6,6'-Dimethyl-2,2'-bipyridine-N-oxide (1)

6,6'-Dimethyl-2,2'-bipyridine (1.97 g, 0.0107 moles) was dissolved in chloroform (10 ml). m-Chloroperbenzoic acid (1.85 g, 0.0107 moles) was dissolved in chloroform (40 ml), and added slowly to the bipyridine solution at 0-5°C. After stirring for two hours at room temperature the solution was extracted twice with saturated sodium hydrogen carbonate solution and three times with water. The chloroform phase was dried and evaporated. The product was purified by flash chromatography.
Yield: 70 %
UV (in ethanol): 270 nm, 250 nm
^{1}H NMR (400 MHz, $CDCl_3$): 2.61 (s, 3 H); 2.62 (s, 3 H); 7.19 (d, 1 H, J = 8 Hz); 7.28 (d, 1 H, J = 5 Hz); 7.69 (t, 1 H, J = 8 Hz); 7.97 (t, 1 H, J = 5 Hz); 8.53 (d, 1 H, J = 8 Hz)

Example 2 (Scheme 1)
6,6'-Dimethyl-4-nitro-2,2'-bipyridine-N-oxide (2)

6,6'-Dimethyl-2,2'-bipyridine-N-oxide (1.50 g, 0.00749 moles) was dissolved in concentrated sulphuric acid (8.0 ml) and fuming nitric acid (6.0 ml) and the mixture was heated at 100°C for four hours. The solution was poured slowly into ice-water and the pH was adjusted to 5.5 with 10 % sodium hydroxide. The product was filtered and dried.
Yield: 53 %
m.p. 160-163°C
UV (in ethanol): 337 nm, 294 nm, 233 nm
^{1}H NMR (400 MHz, $CDCl_3$): 2.62 (s, 3 H); 2.65 (s, 3 H); 7.27 (d, 1 H, J = 8 Hz); 7.75 (t, 1 H, J = 8 Hz); 8.10 (d, 1 H, J = 3 Hz); 8.56 (d, 1 H, J = 8 Hz); 8.93 (d, 1 H, J = 3 Hz)

Example 3 (Scheme 1)
6,6'-Dimethyl-4-ethoxy-2,2'-bipyridine-N-oxide (3)

6,6'-Dimethyl-4-nitro-2,2'-bipyridine-N-oxide (2) (1.0 g, 4.1 mmoles) was added

to sodium ethoxide solution made from (0.19 g, 0.0083 g-atoms) sodium and 25 ml ethanol. The mixture was stirred at 70°C for 30 minutes. After neutralization with concentrated hydrochloric acid the reaction mixture was filtered and evaporated to dryness. The product was purified with flash chromatography (silica, 0→50 % methanol/chloroform).

Yield: 90%

UV (in ethanol): 272 nm, 239 nm

^{1}H NMR (400 MHz, $CDCl_3$): 1.45 (t, 3 H, J = 7.0 Hz); 2.58 (s, 3 H); 2.61 (s, 3 H); 4.14 (q, 2 H, J = 7.0 Hz); 6.84 (d, 1 H, J = 3.4 Hz); 7.20 (d, 1 H, J = 7.7 Hz); 7.54 (d, 1 H, J = 3.4 Hz); 7.70 (t, 1 H, J = 7.7 Hz); 8.65 (d, 1 H, J = 7.7 Hz)

Example 4 (Scheme 1)

6,6'-Dimethyl-4-ethoxy-2,2'-bipyridine (4)

6,6'-Dimethyl-4-ethoxy-2,2'-bipyridine-N-oxide (3) (0.96 g, 3.93 mmoles) was dissolved in chloroform (34 ml). After addition of phosphorus tribromide (3.0 ml) the mixture was refluxed for 1.5 hours. The solution was poured on ice, some chloroform was added and the phases were separated. The chloroform phase was extracted with water. The water phases were combined, made alkaline with sodium hydroxide solution, and extracted with chloroform. Evaporation of the organic phase followed by silica gel chromatography yielded the pure title product.

Yield: 69 %

UV (in ethanol): 286 nm, 243 nm

^{1}H NMR (400 MHz, $CDCl_3$): 1.45 (t, 3 H, J = 7.0 Hz); 2.56 (s, 3 H); 2.62 (s, 3 H); 4.18 (q, 2 H, J = 7.0 Hz); 6.67 (d, 1 H, J = 2.1 Hz); 7.14 (d, 1 H, J = 7.6 Hz); 7.67 (t, 1 H, J = 7.6 Hz); 7.75 (d, 1 H, J = 2.1 Hz); 8.16 (d, 1 H, J = 7.6 Hz).

Example 5

4-Ethoxy-6,6'-bis(bromomethyl)-2,2'-bipyridine (5)

Compound (4) (4,0 mmol) was dissolved in carbon tetrachloride (100 ml). N-bromosuccinimide (9.0 mmol) and a catalytic amount of dibenzoyl peroxide were added and the mixture was refluxed overnight. The mixture was filtered and the solution evaporated to dryness. The pure product was isolated after flash chromato-

graphy separation.
Yield: 19 %
UV (in ethanol): 286 nm, 225 nm
^{1}H NMR (400 MHz, $CDCl_3$): 1.45 (t, J=7.0 Hz); 4.19 (q, J=7.0 Hz); 4.55 (s); 4.60 (s); 6.95 (d, J=2.3 Hz); 7.43 (dd, J=0.9 Hz & 7.6 Hz); 7.77 (t, J=7.6 Hz); 7.90 (d, J=2.3 Hz); 8.35 (dd, J=0.9 Hz & 7.6 Hz)

Example 6 (Scheme 2)
6-Bromo-2-dimethoxymethyl pyridine (14)

6-Bromo-2-pyridinecarboxaldehyde (J.Am.Chem.Soc. 91, (11), 3500 (1970)) (11.38 g, 62.2 mmoles) was dissolved in a mixture of dry methanol (200 ml) and trimethyl orthoformate (26.5 g, 250 mmoles). After addition of p-toluenesulfonic acid monohydrate (250 mg) the mixture was refluxed for 1 h, cooled and neutralized by addition of pyridine (5 ml). Evaporation of solvent and distillation of the product under reduced pressure yielded the pure dimethylacetal as a colorless liquid.
Yield: 96 %
^{1}H NMR (60 MHz, $CDCl_3$): 3.36 (s, 6 H); 5.25 (s, 1 H); 7.46 (m, 3 H)

Example 7 (Scheme 2)
Bis(6-dimethoxymethyl-2-pyridyl)ketone (15)

6-Bromo-2-dimethoxymethyl pyridine (14) (14.2 g, 61 mmoles) was dissolved in dry diethyl ether (200 ml) and cooled down to -70°C in a round bottom three-necked flask equipped with reflux condenser and dropping funnel while a gentle flow of dry argon was passing through the magnetically stirred mixture.
Butyllithium (25.1 ml (2.6M), 65.3 mmoles) was added dropwise and the temperature of the reaction mixture was kept below -60°C. The mixture was stirred for an additional 1 h after accomplised addition, and ethyl chloroformate (4.52 g, 41.7 mmoles) dissolved in dry diethyl ether was introduced at such a rate that the temperature did not exceed -60°C. The yellow suspension was stirred for 45 min at -60°C and then additionally for 15 min at -40°C. The reaction was quenched with methanol (20 ml) whereupon the reaction mixture was poured into saturated aqueous sodium hydrogen carbonate (300 ml). The ether phase was

separated and the aqueous phase was extracted twice with 100 ml of dichloromethane. The combined organic phase was evaporated and coevaporated with toluene which yielded crude title compound (15).
^{1}H NMR (60 MHz, $CDCl_3$): 3.38 (s, 12 H); 5.30 (s, 2 H); 8.18-7.64 (m, 6 H)

Example 8 (Scheme 2)
Bis(6-formyl-2-pyridyl)ketone (16)

Crude bis(6-dimethoxymethyl-2-pyridyl)ketone (15) was dissolved in dioxane (40 ml) and water (25 ml). Concentrated hydrochloric acid (3 ml) was added and the magnetically stirred mixture was boiled for 15 min. The dark solution was then poured into saturated sodium hydrogen carbonate solution (100 ml) and extracted with chloroform (3x100 ml). The residue after evaporation of the combined extracts was filtered through a short silica gel column using 4% EtOH/$CHCl_3$ as a solvent and the fractions containing the product were evaporated. The product was crystallized from hot toluene (100 ml).
Yield: 65% (based on compound 14)
^{1}H NMR (60 MHz, $CDCl_3$): 8.48-8.11 (m, 6 H); 10.07 (s, 2 H)

Example 9 (Scheme 2)
Bis(6-hydroxymethyl-2-pyridyl)ketone (17)

To the compound (16) (2.5 g, 52.5 mmoles) in dry ethanol (50 ml) sodium borohydride (400 mg, 52.5 mmoles) dissolved in dry ethanol (30 ml) was added dropwise at 0°C with gentle stirring. A satisfactory ratio of diol (17)/triol was obtained when about 2/3 of the borohydride had been added. Acetone (20 ml) was added for destroying unreacted reducing agent and the mixture was evaporated, dissolved in chloroform/ethanol 1:1 and extracted with saturated sodium hydrogen carbonate. The organic phase was evaporated, coevaporated with toluene and purified by silica gel column chromatography using EtOH/$CHCl_3$ 1:9 as an eluting solvent.
Yield: 48 %
^{1}H NMR (60 MHz, $CDCl_3$ + CD_3OD): 4.77 (s, 4 H); 7.98-7.27 (m, 6 H)

Example 10 (Scheme 2)
Bis(6-bromomethyl-2-pyridyl)ketone (18)

Bis(6-hydroxymethyl-2-pyridyl)ketone (17) (410 mg, 1.68 mmoles), suspended in dichloromethane (15 ml) was stirred at room temperature in a 50 ml round bottom flask. Phosphorous tribromide (1.82 g, 6.72 mmoles) was added all at once and the mixture was refluxed for 5 min. The cooled mixture was partitioned between saturated sodium hydrogen carbonate and chloroform. The organic phase was collected, evaporated, coevaporated with toluene, and purified by flash chromatography using chloroform as solvent.
Yield: 87 %
UV (in dichloromethane): 282 nm
^{1}H NMR (60 MHz, $CDCl_3$); 4.55 (s, 4 H); 8.16-7.22 (m, 6 H)

Example 11 (Scheme 3)
L-Lysine ethyl ester (26)

Thionyl chloride (5.0 ml, 8.06 g, 68 mmoles) was dropped into 500 ml of ice-cooled dry ethanol. The stirred mixture was kept for 20 min at this temperature and L-lysine hydrochloride (20 g, 109 mmoles) was added.

The mixture was then refluxed for 3 h and concentrated to a volume of about 200 ml. 200 ml of diethylether was added and the crystallized product filtered off.
Yield: 97 % dihydrochloride

Example 12 (Scheme 3)
ω-N-(4-Nitrobenzoyl)-L-lysine ethyl ester (27)

L-lysine HCl (5 g, 27.4 mmoles) dissolved in 50 ml of water was titrated with 5 M NaOH to pH 10.5. 4-Nitrobenzoyl chloride (6.6 g, 36 mmoles) in dioxane (50 ml) and 5 M NaOH were slowly added keeping the vigorously stirred reaction mixture at pH 10.5.

After complete addition and disappearance of the pink color the reaction mixture was acidified with conc. HCl to pH 2 and extracted four times with diethylether. The aqueous phase was concentrated to dryness, coevaporated twice with 200 ml of dry ethanol and suspended in 250 ml of dry ethanol previously treated with 10 ml of thionyl chloride. The mixture was refluxed for 3 h, filtered and evaporated. The residual material was partitioned between saturated sodium hydrogen carbonate and chloroform/ethanol 1:1 and the organic phase was dried over magnesium sulphate yielding a crude product which was purified by flash chromatography using 5% EtOH/chloroform as eluent.
Yield: 12 %
^{1}H NMR (60 MHz, $CDCl_3$): 8.25 (d, 2 H, J=9 Hz); 7.93 (d, 2 H, J=9 Hz); 6.87 (s, broad, 1 H); 4.17 (q, 2 H, J = 7 Hz); 3.30-3.60 (m, 3 H); 1.40-1.75 (m, 8 H); 1.24 (t, 3 H, J = 7 Hz)

Example 13 (Scheme 3)
α-N-(Methoxycarbonylmethyl)-ω-N-(4-nitrobenzoyl)-L-lysine ethyl ester (28)

Compound (27) (0.54 g, 1.7 mmoles) was coevaporated with toluene and dissolved in dry acetonitrile (10 ml). Bromoacetic acid methylester (0.265 g, 1.7 mmoles) was added followed by pulverized dry sodium carbonate (2.0 g). The mixture was refluxed for 3 h.

Filtration of the inorganic salts and evaporation of the acetonitrile gave an oily crude product which was purified by flash chromatography.
Yield: 68 %
^{1}H NMR (60 MHz, $CDCl_3$): 8.25 (d, 2 H, J=9 Hz); 7.93 (d, 2 H, J=9 Hz); 6.63 (s, broad, 1 H); 4.13 (q, 2 H, J = 7 Hz); 3.68 (s, 3 H); 3.30-3.60 (m, 3 H); 1.40-1.75 (m, 7 H); 1.24 (t, 3 H, J = 7 Hz).

Example 14

GENERAL PROCEDURE FOR THE SYNTHESIS OF REACTIVE LIGAND OR CHELATE EMPLOYING DERIVATIVES OF α, ω DIAMINOACIDS AS STARTING MATERIAL

a) The appropriate dihalomethyl bipyridine (1 mmol) in dry acetonitrile (10 ml) is reacted with (1 mmol) of compound (28) in the presence of 2 g powderized

dry sodium carbonate at room temperature with vigorous stirring. After overnight stirring the resulting mixture composed of unreacted dihalomethyl derivative, monohalomethyl diester and symmetrical tetraester is evaporated, coevaporated with toluene and flash chromatographed to obtain pure monohalomethyl diester.

b) The monohalomethyl diester (0.2 mmoles) obtained is coevaporated with dry acetonitrile, dissolved in 3 ml of acetonitrile, and 1 g of powderized sodium carbonate is added followed by iminoacetic acid diethyl ester (0.25 mmoles). The mixture is refluxed overnight, filtered and evaporated. The desired functionalized tetraester is obtained after short column chromatography.

c) When compound (28) is used as a starting material in Example 14 a) the nitro group has to be reduced to amino prior to ester group hydrolysis and chelate formation. This is performed in the following way:

Solid sodium borohydride (0.3 mmoles) is added to the mixture of the respective product from Example 14 b) followed by 0.2 g palladium on carbon (10%) in 5 ml of dry ethanol. The mixture is stirred at room temperature for 10 min and partitioned between saturated sodium hydrogen carbonate and chloroform. The evaporated organic extracts are flash chromatographed to yield the respective tetraester containing a reactive amino group.

Example 15 (Scheme 1)
4-Ethoxy-6-(N-(methoxycarbonylmethyl)-N-(1-(5-(p-nitrobenzamido))-1-(ethoxycarbonyl)-pentyl)-aminomethyl)-6'-bromomethyl-2,2'-bipyridine (6)

This compound was synthesized using the general procedure from Example 14a when 4-ethoxy-6,6'-bis(bromomethyl)-2,2'-bipyridine (5) and modified iminodiacetic acid ester (compound 28) were used as starting materials.
Yield: 38 %
UV (in ethanol): 286 nm, 220 nm
^{1}H NMR (400 MHz, $CDCl_3$): 1.30 (t, J=7 Hz); 1.45 (t, J=7 Hz); 1.55-1.68 (m); 1.75-1.83 (m); 3.45 (m); 3.50 (m); 3.55 (d, J=15 Hz); 3.64 (s); 3.66 (d, J=15 Hz); 3.94 (d, J=15 Hz); 4.03 (d, J=15 Hz); 4.18 (q, J=7 Hz); 4.19 (q, J=7 Hz);

4.58 (s); 6.83 (s); 7.45 (s); 7.62 (d, J=7 Hz); 7.76 (t, J=7 Hz); 8.00 (d, J=9 Hz); 8.25 (d, J=9 Hz); 8.28 (d, J=7 Hz); 8.35 (s)

A symmetrical disubstituted tetraester (7) was also obtained in 26 % yield.

Example 16 (Scheme 1)
4-Ethoxy-6-(N-(methoxycarbonylmethyl)-N-(1-(5-(p-nitrobenzamido))-1-(ethoxycarbonyl)-pentyl)-aminomethyl-6'-(N,N-bis(ethoxycarbonylmethyl)aminomethyl-2,2'-bipyridine (8)

This compound was synthesized following the general procedure for tetraester synthesis from Example 14b using compound (6) and diethyl iminodiacetate as starting materials.
Yield: 62 %
UV (in ethanol): 287 nm, 222 nm
^{1}H NMR (400 MHz, $CDCl_3$): 1.26 (t, J=7 Hz); 1.28 (t, J=7 Hz); 1.46 (t, J=7 Hz); 1.59 (m); 1.76 (m); 3.35 (m); 3.49 (m); 3.58 (d, J=15 Hz); 3.65 (s); 3.66 (s); 3.67 (s); 3.71 (d, J=15 Hz); 3.96 (d, J=15 Hz); 4.05 (d, J=15 Hz); 4.09 (s); 4.14 (q, J=7 Hz); 4.17 (q, J=7 Hz); 6.88 (s); 7.15 (d, J=2 Hz); 7.50 (d, J=7 Hz); 7.75 (d, J=7 Hz); 7.83 (d, J=2 Hz); 8.01 (d, J=9 Hz); 8.24 (d, J=7 Hz); 8.26 (d, J=9 Hz)

Example 17 (Scheme 1)
4-Ethoxy-6-(N-(methoxycarbonylmethyl)-N-(1-(5-(p-aminobenzamido))-1-(ethoxycarbonyl)-pentyl)-aminomethyl)-6'-(N,N-bis(ethoxycarbonylmethyl)aminomethyl)-2,2'-bipyridine (9)

This compound was synthesized using the general procedure for nitrogroup reduction from Example 14c having compound (8) as a starting material.
Yield: 71 %
UV (in ethanol): 286 nm, 222 nm
^{1}H NMR (400 MHz, $CDCl_3$): 1.26 (t, J=7 Hz); 1.28 (t, J=7 Hz); 1.46 (t, J=7 Hz); 1.60 (m); 1.75 (m); 3.41 (m); 3.44 (m); 3.54 (d, J=15 Hz); 3.62 (d, J=15 Hz); 3.64 (s); 3.69 (s); 3.92 (d, J=15 Hz); 4.01 (s); 4.04 (d, J=15 Hz); 4.16 (q, J=7 Hz); 4.17 (q, J=7 Hz); 6.30 (s); 6.64 (d, J=9 Hz); 7.16 (d, J=2 Hz); 7.50 (d, J=7 Hz); 7.63 (d, J=9 Hz); 7.78 (t, J=7 Hz); 7.80 (d, J=2 Hz); 8.24 (d, J=7 Hz)

Example 18 (Scheme 1)
4-Ethoxy-6-(N-(carboxymethyl)-N-(1-(5-(p-aminobenzamido))-1-carboxypentyl)amino-methyl)-6'-(N,N-bis(carboxymethyl)-aminomethyl)-2,2'-bipyridine and its europium (10) and terbium (11) chelates, respectively

Compound (9) (0.25 mmol) dissolved in acetone (10 ml) was treated at 20°C with sodium hydroxide (1 M, 10 ml) for 1 hour. The alkaline solution was neutralized with concentrated hydrochloric acid and most of the salts were precipitated by addition of acetone (20 ml). The salts were filtered off and the organic solvent was evaporated. The residual aqueous solution of tetra acid was adjusted to pH 5.0 and divided into two equal portions. To each was added an equivalent amount of $EuCl_3$ or $TbCl_3$ respectively in the form of a solution in water (5 ml) and both mixtures were stirred at this pH for 30 min whereupon the pH's were increased to 8.0. Some precipitates were formed in both reactions and were filtered off. Most of the water was evaporated under reduced pressure and both the europium (10) and the terbium (11) chelate were isolated as the solids material after precipitation from acetone (50 ml).

Example 19 (Scheme 1)
Isothiocyano chelates

To the amino chelate (10) or (11) (100 mg) dissolved separately in 5 ml of water and vigorously stirred, thiophosgene (80 µl) dissolved in chloroform (3 ml) was added at once and the mixtures were stirred at RT for 1 h.

The water phases were separated, extracted with chloroform (3 x 3 ml) and concentrated to a volume of 0.5 ml. Addition of ethanol (10 ml) precipitated isothiocyano functionalized europium chelate (12) and terbium chelate (13) quantitatively as white solids. The TLC (Acetonitrile/H_2O 4:1) and fluorescence developing with acetonylacetone /EtOH (1:20) showed only a single product which was negative to a fluorescamine test for free amines.

Example 20 (Scheme 2)
6-Bromomethyl-6'-(N,N-bis(ethoxycarbonylmethyl)aminomethyl)-2,2'dipyridyl ketone (19)

The monobromomethyl diester (19) was synthesized starting from compound (18) and one molecular equivalent of iminodiacetic acid diethyl ester, in a reaction analogous to that of Example 14 a. Yield: 62 %.

^1H NMR (60 MHz, $CDCl_3$): 1.21 (t, 6 H), 3.60 (s, 4 H), 4.08 (s, 2 H), 4.15 (q, 4 H), 4.58 (s, 2 H), 7.22-8.15 (m, 6 H).

In the same reaction the symmetrical tetraester (20) was obtained and isolated with a 21 % yield.

Example 21 (Scheme 2)
6-(N-(methoxycarbonylmethyl)-N-(1-(5-(p-aminobenzamido)-1-(ethoxycarbonyl)-pentyl)aminomethyl-6'-(N,N-bis(ethoxycarbonylmethyl)aminomethyl)-2,2'dipyridyl ketone (21)

a) The nitro group in compound (28) (2.1 g, 5 mmol): 20 ml of dry methanol was reduced with sodium borohydride (10 mmol) in the presence of palladium on carbon (10 %, 0.2 g). The reaction mixture was stirred at RT for 15 min and partitioned between saturated sodium hydrogen carbonate and chloroform. The evaporated organic phase was flash chromatographed to yield the oily product (29 in scheme 3) pure by TLC and NMR. Yield 84 %.

^{1}H NMR (400 MHz, $CDCl_3$); 1.25 (t, 3 H), 1.45 - 1.80 (m, 6 H) 3.39 - 3.48 (m, 3 H), 3.57 (s, 2 H), 3.70 (s, 3 H), 4.17 (q, 2 H), 6.28 (t, 1 H, broad), 6.64 (d, 2 H, J = 8.7 Hz), 7.63 (d, 2 H, J = 8.7 Hz).

b) compound (19) was reacted with the above reduced derivative of (28) (2 eq) under the conditions described in Example 14 b.

Filtration, evaporation of solvent and flash chromatography gave the product free from minor contaminations of isomeric tetraester which resulted from coupling via aromatic amino group. Yield 79 %.

^{1}H NMR (400 MHz, $CDCl_3$): 1.20 - 1.30 (m, 9 H), 1.45 - 1.80 (m, 6 H), 3.50 - 3.67 (d.d., 2 H, J = 17.7 Hz), 3.64 (s, 4 H), 3.70 (s, 3 H), 4.01 (s, 2 H), 3.89-4.05 (d.d., 2 H), 4.12 - 4.20 (m, 6 H), 6.28 (t, 1 H, broad), 6.64 (d, 2 H, J = 8.7 Hz), 7.20 - 7.80 (m, 6 H), 7.63 (d, 2 H, J = 8.7 Hz)

Example 22 (Scheme 2)
6-(N-(carboxymethyl)-N-(1-(5-(p-aminobenzamido)-1-carboxypentyl)aminomethyl-6'-(N,N-bis(carboxymethyl)aminomethyl)-2,2'dipyridyl ketone, its europium (22) and terbium chelates (23) and their isothiocyano derivatives (24) and (25)

Hydrolysis of compound (21), formation of europium (22) and terbium (23) chelates and their reactions with thiophosgene to form the isothiocyano

derivatives (24) and (25), respectively, were performed as described in Examples 18 and 19. Both final products were stored in the form of ethanol precipitates.

Example 23

M 13 phage sequencing primer and its labeling with lanthanide chelates

The hexadecamer oligonucleotide of M 13 phage sequencing primer was synthesized using Pharmacia Gen Assembler solid phase nucleic acid synthesizer (Pharmacia, Sweden) following the recommended conditions. When the oligonucleotide synthesis had been accomplished the detritylated compound was subjected to the reaction with 1,1'-carbonyldiimidazole and diaminohexane according to Nucleic Acids Research 14, 7987-7994 (1986).

The deprotection of such a synthesized primer was made under standard conditions and was followed by preparative FPLC. The isolated 5'-amino modified hexadecamer was pure as shown by chromatographic and electrophoretic analysis.

Four portions of the above oligonucleotide (5 OD each) were reacted at RT for 6 h with lanthanide chelates 12, 13, 24 and 25, (3 mg) respectively, each reaction being carried out in a solution containing sodium carbonate (0.2 M, 1.0 ml).

The reactions were monitored by FPLC, and labeled products from each reaction were separated in ion-pair mode using the reverse phase FPLC system. The purified sequences were characterized by their fluorescent spectra which were found to be identical to the unbound labels.

Moreover, the four differently labeled primers did not show any differences in their electrophoretic mobilities when run under the real sequencing conditions on a 20 % acrylamide gel. This reflects the fact that all labels employed are indeed very closely structure related. No differences were found even when electrophoresis was used for running free labels.

It should be noted also that despite the rather high gel temperature (50°C), and despite the presence of additional complexing agents (EDTA) in the electrophoretical buffer, there was no sign of instability of any of the lanthanide chelates.

Example 24

Sequencing reactions

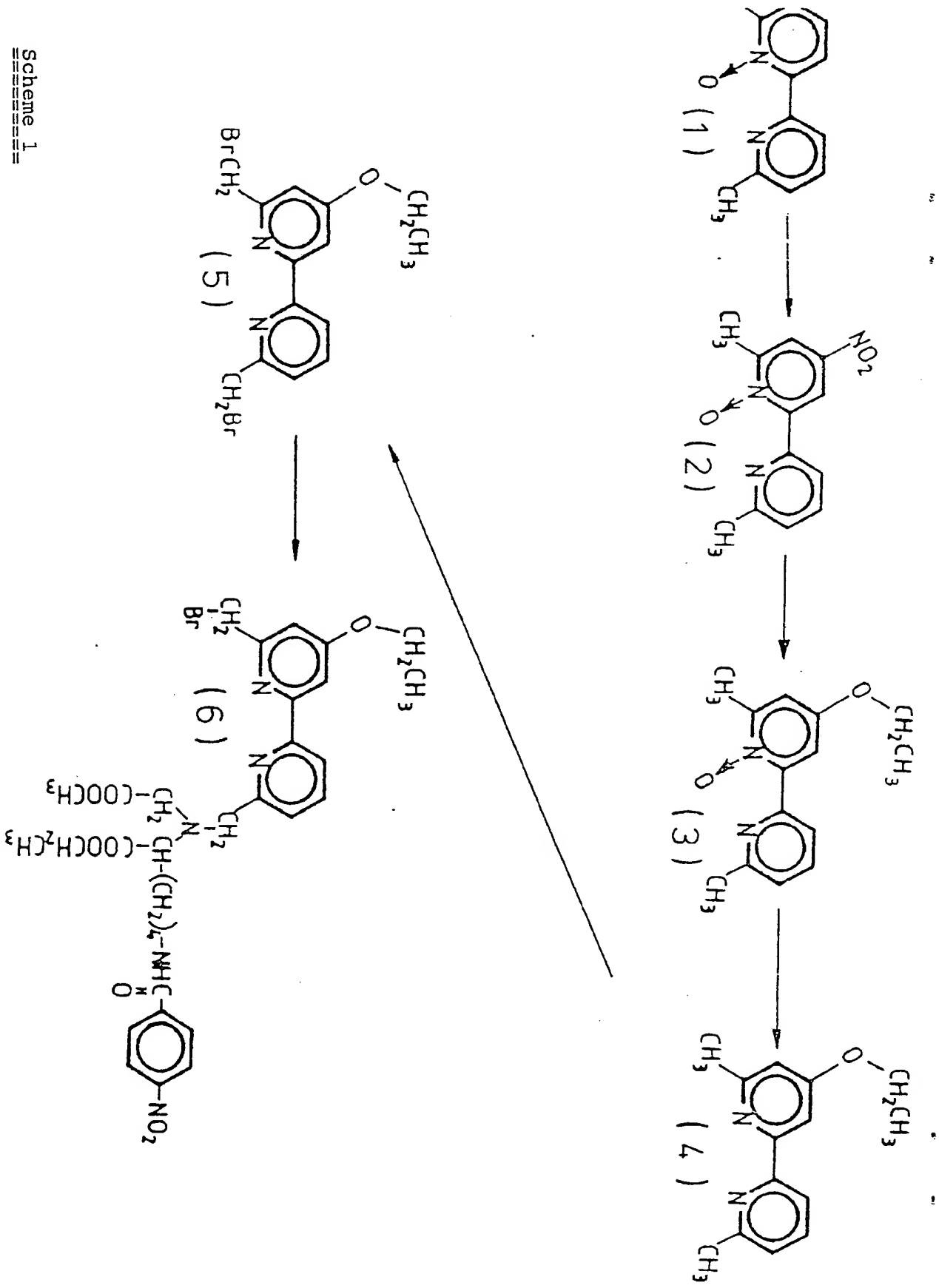
To prove the applicability of the invented markers as a tag for nucleic acids sequencing, all differently labeled oligonucleotides were used as sequencing primers in the sequence analysis performed by standard methods (Proc. Natl. Acad. Sci. USA 74, 5463-5467), utilizing α-^{32}P dCTP as a radiolabel. In each case only one dideoxynucleotide (ddATP) was used and the resulting mixtures were run parallelly on the sequencing gel.

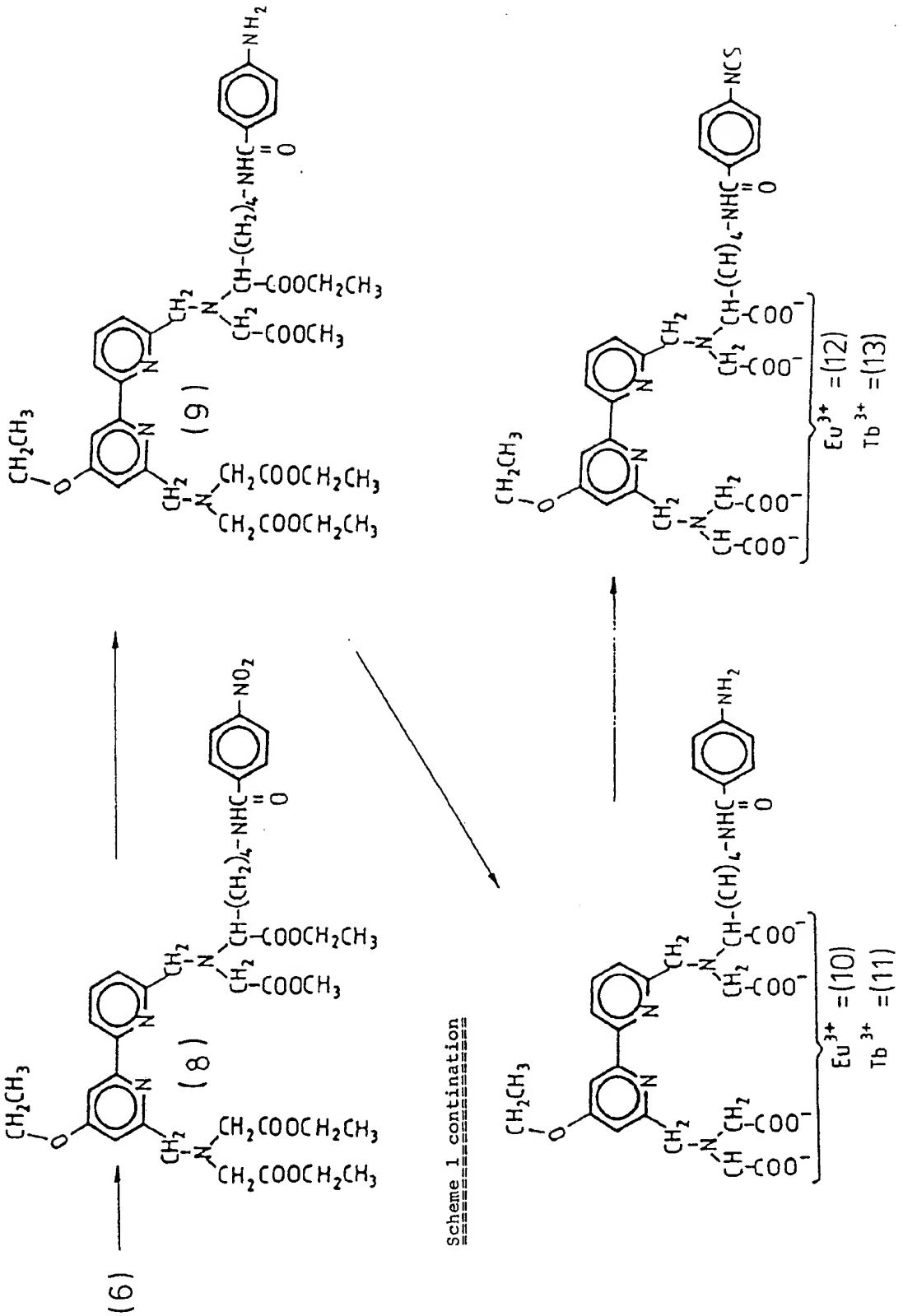
All primers gave the same sequencing pattern indicating that the derivatized primers retain their ability to hybridize specifically to the complementary strand.

No evidence was found that the bands obtained differ in their mobilities.

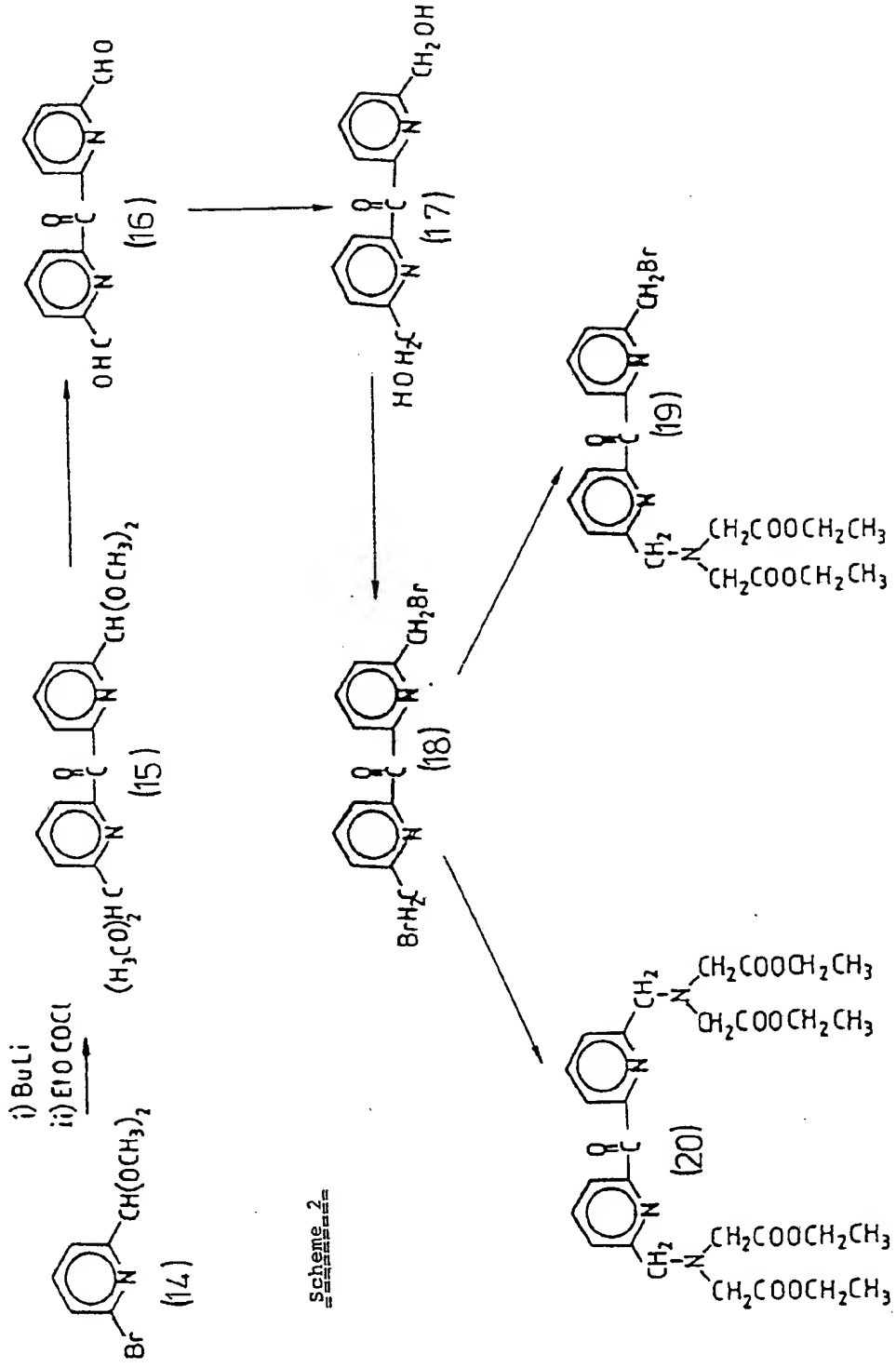
The appropriate bands were cut out and the presence of europium $^{+3}$ was found using Arcus Fluorometer (Wallac Oy, Finland) and enhancement solution (Wallac Oy).

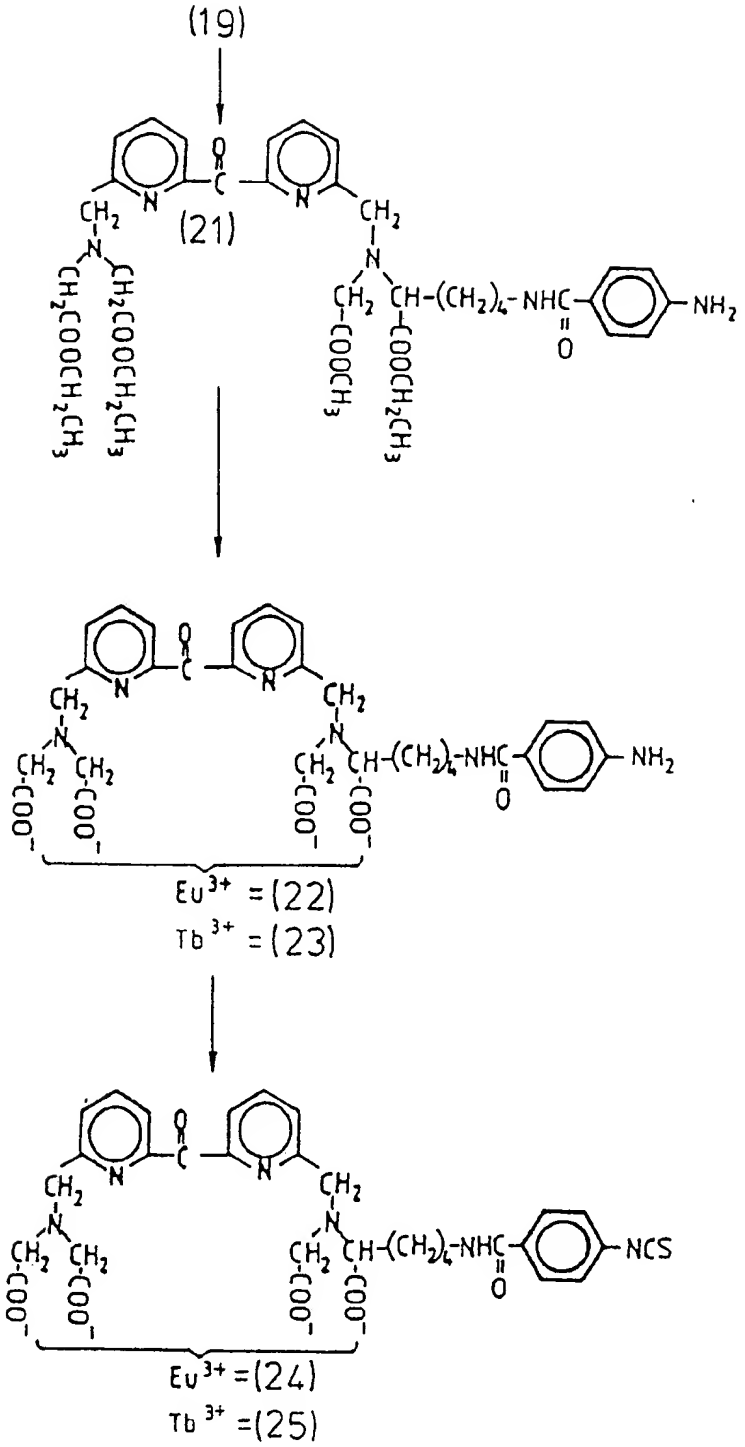
Scheme 1



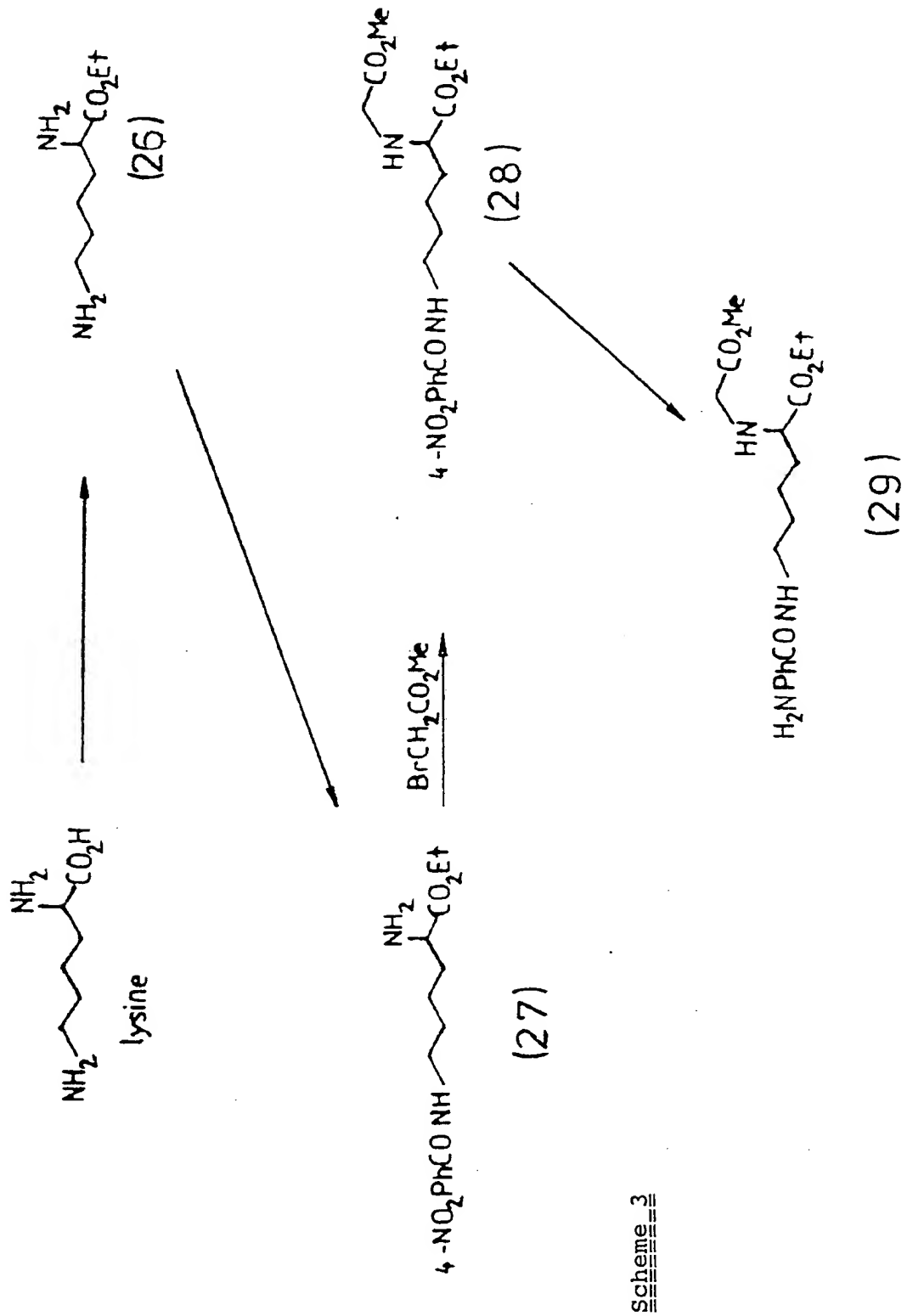


Scheme 1 contination


i) BuLi
ii) EtO COCl
(14)
(15)
(16)
(17)
(18)
(19)
(20)
Scheme 2



Scheme 2 contination


NH2
CO2Et
NH2
(26)
HN
CO2Me
CO2Et
4-NO2PhCONH
(28)
NH2
CO2H
NH2
lysine
BrCH2CO2Me
NH2
CO2Et
4-NO2PhCONH
(27)
HN
CO2Me
CO2Et
H2NPhCONH
(29)

Scheme 3

Claim

Method for simultaneous real time fluorometric sequence analysis of nucleic acid fragments of different lengths and four different terminals, the fragments being labeled with different fluorescent labels for the respective terminating bases, said method comprising

- electrophoretically separating the mixture of the four labeled fragments along a single path in a separation medium,
- focusing fluorometric excitation light on a predetermined spot in said medium through which spot the fragments move,
- detecting photons emitted by the respective labels upon excitation by said light when the fragments move through said spot, and
- determining the nucleic acid sequence on the basis of the photons emitted by the different labels,

characterized in

that the fragments are labelled with four different labels comprising the same two lanthanides and the same two ligands as follows:

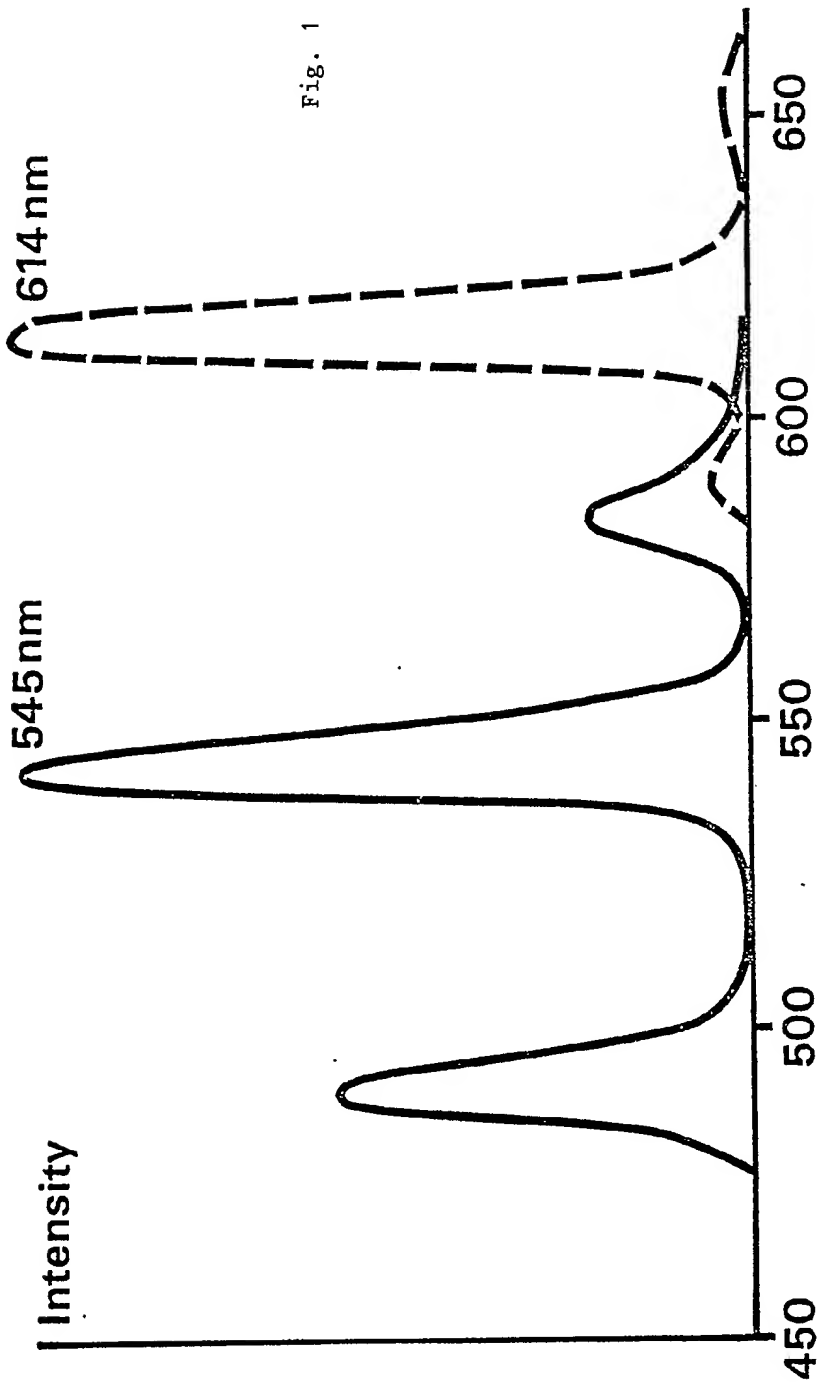
label 1 = ligand 1 + lanthanide 1
label 2 = ligand 2 + lanthanide 1
label 3 = ligand 1 + lanthanide 2
label 4 = ligand 2 + lanthanide 2

where ligands 1 and 2 have different excitation wavelengths and lanthanides 1 and 2 have different emission wavelengths,

that the excitation light is generated in the form of light pulses alternating between the different excitation wavelengths of the ligands, and

that the photons emitted by the labels are detected periodically for the different emission wavelengths of the lanthanides.

Fig. 1



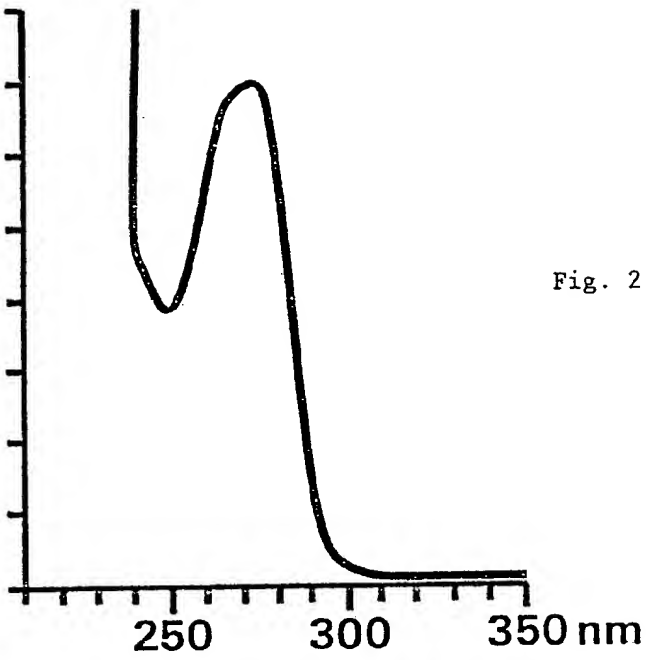


Fig. 2

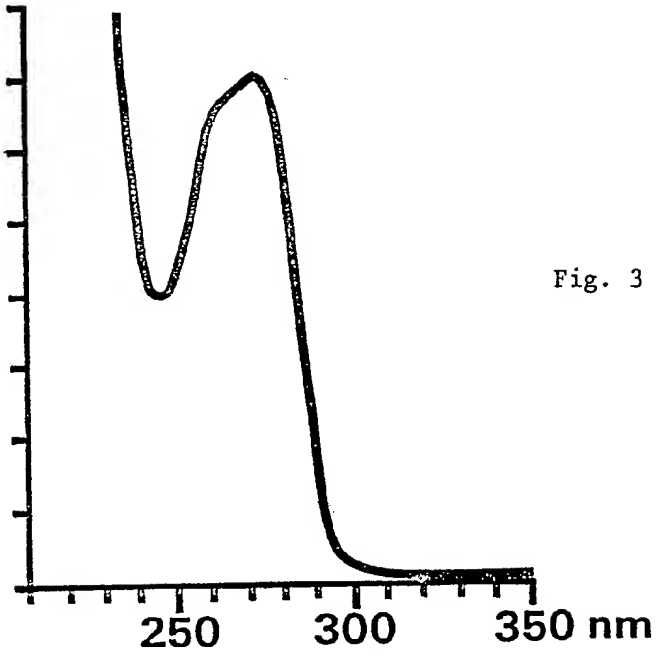


Fig. 3

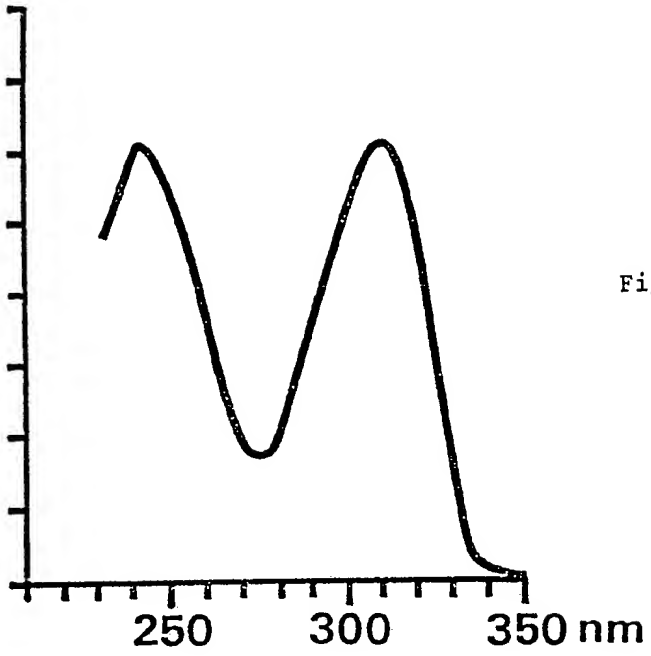


Fig. 4

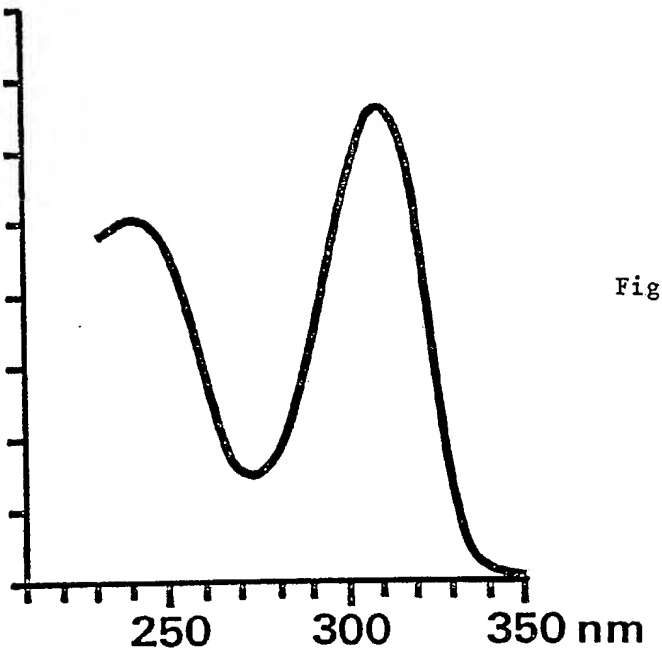


Fig. 5

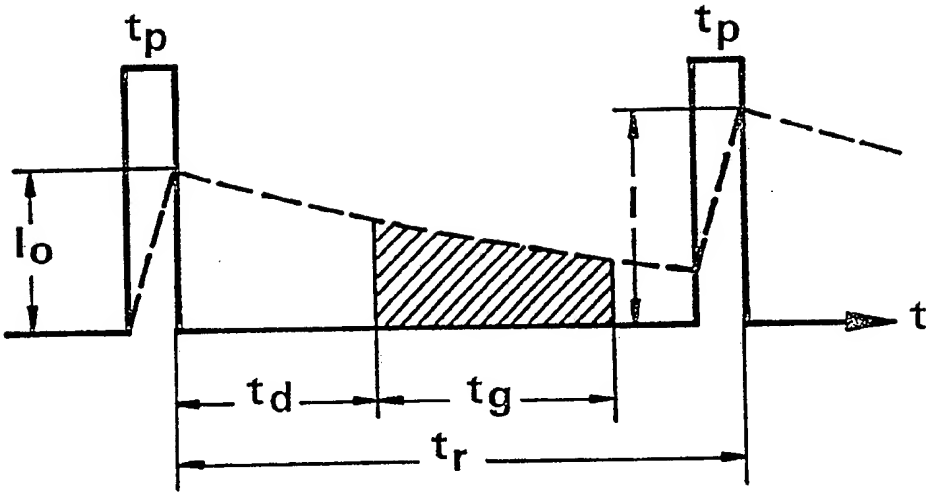


Fig. 6

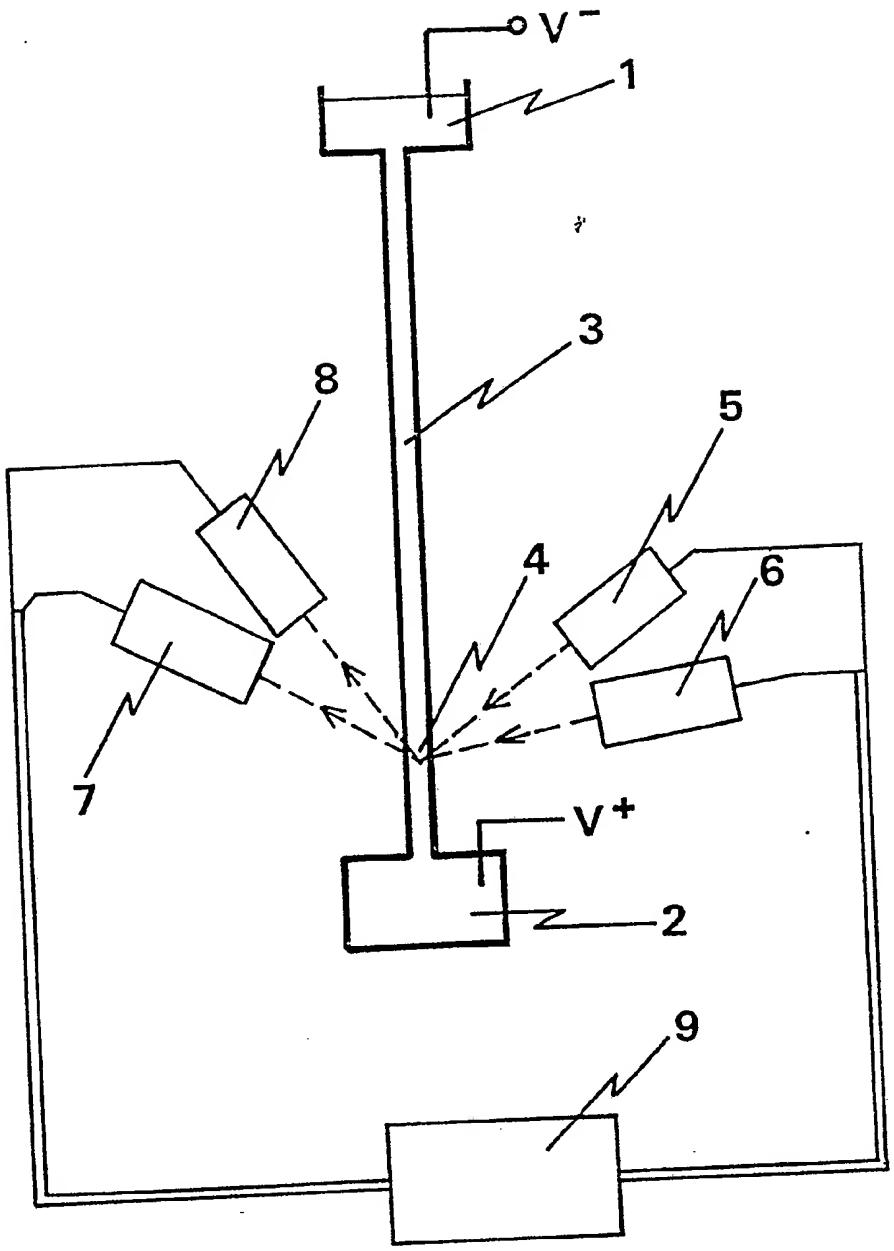


Fig. 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE89/00378

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC [4]

C 12 Q 1/68, G 01 N 33/58, G 01 N 26/27

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC 4	C 12 Q; G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

SE, NO, DK, FI classes as above.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	EP, A2, 0 252 683 (E.I. DU PONT DE NEMOURS AND COMPANY) 13 January 1988 See page 9 line 2 - page 11 line 51	1
A	Nature, Vol 321, 12 June 1986, Lloyd M.Smith et al: "Fluorescence detection in automated DNA sequence analysis", p 674-679 See abstract, figures 1-3	1
A	Nucleic Acids Research, Vol 15, No 11, 1987, Wilhelm Ansorge et al: "Automated DNA sequencing: ultrasensitive detection of fluorescent bands during electrophoresis", p 4593-4601 See abstract, p 4600 line 23 - p 4601 line 21	1
A	EP, A2, 0 268 406 (AMERSHAM INTERNATIONAL) 25 May 1988	1
A	DE, A1, 3 501 306 (CALIFORNIA INSTITUTE OF	1

* Special categories of cited documents: [10]

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1989-08-25	1989 -08- 28
International Searching Authority	**Signature of Authorized Officer**
Swedish Patent Office	Mikael G:son Bergstrand

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	TECHNOLOGY) 25 July 1985 See abstract and figures	